

Production and Characterization of Stable Amphotericin-Resistant Amastigotes and Promastigotes of *Leishmania mexicana*

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The sensitivities of *Leishmania mexicana* amastigote and promastigote forms to amphotericin B were investigated in vitro and found to be strongly influenced by the culture media used. When differences in culture media were minimized, there was no significant difference in the 50% inhibitory concentration values between the two life cycle stages. Stable amphotericin B-resistant amastigote and promastigote lines were produced by the application of increasing drug pressure to long-term cultures. Lines capable of growth in concentrations of amphotericin B lethal to normal parasites were produced. Compared to normal parasites, these amphotericin-resistant lines showed marked differences in membrane sterol compositions, with very high levels of 4,14-dimethyl-cholesta-8,24-dienol and other methyl sterols. They also showed a consistent morphological feature, the presence of multilamellar membrane-like material in the flagellar pocket, revealed by transmission electron microscopy. Amphotericin-resistant parasites were capable of infecting BALB/c mice, but the resulting lesion growth was slower than that after infection with normal parasites. However, unlike normal parasites, the amphotericin-resistant parasites were unaffected by experimental chemotherapy with amphotericin B. These results show that amphotericin B resistance could arise as a result of increased clinical use of amphotericin B therapy.

Current control of leishmaniasis is reliant mainly on chemotherapy of patients, but unfortunately the range of drugs available is relatively limited (9, 40). The most widely used drugs remain the pentavalent antimonials, sodium stibogluconate and meglumine antimoniate, but drug resistance is becoming an increasing problem in countries of leishmaniasis endemicity (39). The introduction of miltefosine, the first oral antileishmanial drug, is an encouraging new development, and this drug may come to replace the antimonials as the first-line drug in due course (36), although there are safety concerns with women of childbearing age (10). However, when unresponsiveness to antimonials is observed clinically, the usual second-line drug is amphotericin B (AMB), given either as a free drug preparation solubilized in deoxycholate or as lipid preparations. The use of AMB is now becoming widespread in Bihar (India) and Nepal (35), and previous reservations about the use of this potentially toxic drug have been alleviated (38). Experimental studies have also shown the possibility of reducing toxicity by heat treating AMB (4, 28), and several groups are developing new formulations of AMB (1, 12, 13, 23, 24, 33). Thus, it has recently been suggested that AMB should replace antimonials as the drug of choice in Bihar (37). In addition, patients coinfecting with *Leishmania infantum* visceral leishmaniasis and human immunodeficiency virus in resource-rich countries are usually treated with liposome-entrapped AMB (8, 27). Guidelines for treatment with AMB and other antileishmanial drugs have been improved, which, in addition to

better treatment of individual patients, should lead to fewer relapses and therefore a decreased risk of secondary resistance. However, no protocols specifically designed to avoid the emergence of drug resistance are in operation (6), and the emergence of AMB resistance is probably a matter of time unless preventative measures are taken. A further pressure is the insistence of some dog owners in Europe on the use of AMB to treat *L. infantum*-infected dogs. These dogs often remain infected despite repeated treatment and, therefore, provide ideal conditions for selection of drug resistance, since the dogs are part of the transmission cycle of *L. infantum* to humans (2). Also, secondary nonresponsiveness has already been reported in immunocompromised patients coinfecting with human immunodeficiency virus and visceral leishmaniasis who relapsed after treatment with liposomal AMB (22). Further, in a series of isolates of *L. infantum* obtained from 18 immunocompromised adults, one that was from a patient who relapsed six times following AMB treatment had a 90% inhibitory concentration (IC₉₀) for AMB that was 10 times higher than that of the original isolate (11).

Experimental evidence that AMB resistance is possible in clinical leishmaniasis has been further suggested by two laboratory studies that generated AMB-resistant lines relatively easily. Exposure of *L. donovani* promastigotes (insect vector stages) to AMB in culture generated lines in which the IC₅₀ was raised from 0.1 μ M to 1.9 μ M (26). Similarly, studies of *L. tarentolae*, a nonpathogenic parasite of geckos, generated two mutant lines with IC₅₀ values of 0.5 μ M and 7.5 μ M (34). The mechanisms underlying such resistance to AMB are not properly understood, and these previous studies also suffer from the disadvantage that they were performed with promastigote stages rather than the pathogenic amastigote stages responsible for human leishmaniasis. Therefore, the current study, in which AMB resistance was induced and investigated for both

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promastigote and amastigote stages of *L. mexicana*, was undertaken. Here we report biochemical and morphological analyses of AMB-resistant *L. mexicana* and the evaluation of these parasites in experimental chemotherapy.

MATERIALS AND METHODS

Parasites used. *L. mexicana* M379 (MNYC/BZ/62/M379) was used in all experiments.

In vitro culture. Previously described methods were used for the routine culture of *L. mexicana* amastigotes (3) and promastigotes (41). The standard culture medium for amastigotes was Grace's medium (Life Technologies) supplemented with 20% fetal calf serum (FCS; Life Technologies), basal medium Eagle vitamins (Life Technologies), and 25 μ g gentamicin sulfate per ml (Sigma), with the pH adjusted to 5.5. The standard culture medium for promastigotes was Medium 199 (Life Technologies) supplemented with 10% FCS, basal medium Eagle vitamins, and 25 μ g gentamicin sulfate per ml, pH 7.2. These culture conditions were varied for specific experiments, as described in Results.

Source of AMB. AMB was purchased from Sigma Chemical Co. (A-2942) as a 250- μ g/ml solution solubilized in sodium deoxycholate.

Drug sensitivity assays. AMB was directly added to cultures at the desired concentrations, and parasites were maintained under in vitro culture conditions for the desired period of time. Cell density was estimated daily by hemocytometer counting. Growth was expressed as a percentage of that shown by drug-free control cultures after 3 days of incubation in vitro and was used for calculations of IC₅₀ values with Graft version 4 software (EriThacus Software, Ltd.).

Selection of AMB-resistant parasites. Drug-resistant lines were generated by subpassaging promastigotes and amastigotes in media containing increasing concentrations of AMB, beginning at 0.125 μ g/ml and increasing in stepwise increments of 0.125 μ g/ml. At each concentration of AMB, parasites were maintained until a growth rate equivalent to that of the control cultures was exhibited; typically, this was achieved in three to four passages, after which the concentration was increased.

Sterol analyses. Sterol analyses were performed as previously described (17, 20). The trimethylsilyl derivatives of free sterols were analyzed by gas chromatography-mass spectrometry, using an HP 6890/5992A gas chromatography system (Agilent Technologies) and 5 α -cholestanol as an internal standard.

Transmission electron microscopy. Individual samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 45 min at 26°C, the fixative was discarded and replaced with 0.1 M cacodylate buffer, pH 7.4, and the samples were stored at 4°C until being processed further. Samples were postfixed for 15 min in 1% (wt/vol) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, at room temperature and then washed in buffer for 15 min. These samples were then dehydrated through an ethanol series and transferred to propylene oxide for 15 min, propylene oxide-Epon-Araldite resin (1:1) for 30 min, resin alone for 1 h, and resin again for 3 h. Samples were then embedded in fresh resin and polymerized at 80°C for 48 h. Ultrathin sections were cut at 90 nm, picked up on 200-mesh hexagonal copper grids, stained for 20 min with uranyl acetate, washed in distilled water, and then stained for 5 min in lead citrate. Finally, the sections were washed with 0.02 M NaOH and then with distilled water and dried. Sections were observed with a Philips CM10 transmission electron microscope.

Experimental chemotherapy. Female BALB/c mice were infected by the injection of 10⁶ stationary-phase promastigotes, either wild type or AMB resistant, into their shaven rumps. When small lesions developed, the mice were treated by intravenous injection of AMB into the tail vein at 1 mg/kg on six occasions over a 2-week period. Lesion development was monitored weekly before and after chemotherapy. Lesion width and length were measured with Vernier calipers. All procedures involving animals were performed in accordance with United Kingdom government (Home Office) and European Commission regulations.

RESULTS

Assay systems. To establish the basic culture conditions for the determination of drug sensitivity and the generation of drug-resistant lines, a number of preliminary studies were carried out. To test the stability of AMB under culture conditions, 0.25 μ g/ml of AMB (previously shown to result in significant inhibition of growth of wild-type cells) was added to each culture medium, which was then preincubated for various periods of time (0 to 6 days) before the addition of parasites,

whose growth was then subsequently monitored. Results showed that drug activity was still apparent after a 3-day preincubation, but after a 6-day preincubation there was little remaining activity against either promastigotes or amastigotes. Based on this, all determinations of IC₅₀ were carried out after 3 days of exposure to drug. It was also concluded that weekly subpassage of parasites, following exposure to sublethal concentrations of AMB, would be suitable for the selection of drug-resistant lines, as this would enable sufficient time for exposure to AMB, selective killing of sensitive parasites, and recovery of cultures.

The influence of culture conditions on IC₅₀ determinations was also investigated. Amastigotes cultured under standard conditions at pH 5.5 and 32°C in Grace's medium with 20% FCS showed an IC₅₀ of 0.20 \pm 0.025 μ g/ml (mean \pm standard error), whereas promastigotes cultured at pH 7.2 and 26°C in Medium 199 with 10% FCS showed an IC₅₀ of 0.04 \pm 0.0003 μ g/ml. However, when determinations of drug sensitivity for both amastigotes and promastigotes were carried out under conditions of Grace's medium, 15% FCS, and pH 5.5, with a starting density of 2 \times 10⁶ cells/ml but at different temperatures, the sensitivities of amastigotes and promastigotes to AMB were not significantly different (IC₅₀s of 0.14 \pm 0.01 and 0.16 \pm 0.001 μ g/ml, respectively). These data also suggested that the concentration of FCS in culture medium was a major factor affecting the activity of AMB. This was examined in further detail by a series of experiments exposing amastigotes and promastigotes to AMB under a range of FCS concentrations. These experiments showed that AMB was less effective in the presence of higher concentrations of serum (data not shown).

Generation of drug-resistant lines. In order to generate AMB-resistant lines of *L. mexicana*, lesion amastigotes were isolated from infected BALB/c mice and used to establish axenic cultures of amastigotes and promastigotes. These were then cultured in the presence of gradually increasing concentrations of AMB, beginning at 0.125 μ g/ml, a sublethal concentration of AMB, to generate drug-resistant lines. To confirm experimental reproducibility, two independent lines of amastigotes (AR1 and AR2) and promastigotes (PR1 and PR2) were generated, all of which were capable of growth in concentrations of AMB (\geq 3.75 μ g/ml) that were lethal to normal cells (Table 1). To test for the stability of drug resistance, selection was relaxed in two of these lines, which were cultured in the absence of AMB for 19 passages before their AMB sensitivities were retested. These lines remained highly resistant to AMB and capable of growing in the presence of lethal AMB concentrations (Table 1). These data indicate that resistance to AMB was not readily reversed.

Sterol analyses of drug-resistant lines. The putative mode of action of AMB is that the drug binds to sterols present in the surface membranes of target cells; this results in the formation of pores, and the cells are killed by osmotic lysis (31). Therefore, the sterol compositions of wild-type and AMB-resistant *L. mexicana* lines were investigated. Analyses of drug-resistant and normal, wild-type *L. mexicana* lines revealed dramatic differences in sterol composition (Table 2). Typically, the major sterol found in promastigotes of *L. mexicana* is ergosta-5,7,24(24¹)-trienol (7), which, in the current study, contributed approximately 85% of the total sterol. In the cultured amasti-

TABLE 1. IC₅₀ values for AMB-resistant lines of *L. mexicana* promastigotes and amastigotes

Line ^a	Final concn of AMB (μg/ml) in culture medium	IC ₅₀ (μg/ml [mean ± SE])	Culture conditions ^b
Amastigote control		0.2 ± 0.025	Grace's, 20% FCS
AR1	5.0	2.22 ± 0.29	Grace's, 20% FCS
AR1 grown for 19 passages without AMB	2.75	1.4 ± 0.24	Grace's, 20% FCS
AR2	3.75	ND ^c	Grace's, 20% FCS
Promastigote control		0.16 ± 0.015	Grace's, 10% FCS
PR1	6.25	2.6 ± 0.3	Grace's, 10% FCS
PR1 grown for 19 passages without AMB	4.0	2.52 ± 0.25	Grace's, 10% FCS
PR2	5.5	ND	Grace's, 10% FCS

^a AR1 and AR2 were two independently generated amastigote lines; similarly, PR1 and PR2 were independently generated promastigote lines.

^b Grace's, Grace's medium.

^c ND, not determined.

gotes, the major sterols were a mixture of ergosta- and stigmasta-5,7,24(24¹)-trienols, which is in agreement with the composition seen for amastigotes isolated from J774 cells (18). In contrast, these sterols were undetectable in AMB-resistant parasites, which contained instead high levels of methylcholesta sterols. These included sterols that are found at low levels as normal intermediates in the early part of the sterol biosynthetic pathway (4,14,dimethyl-cholesta-8,24-dienol and lanosta-8,24-dienol) but also 4,14,dimethyl-cholesta-7,24-dienol, which is not part of the normal biosynthetic pathway.

Characterization of drug-resistant lines. AMB-resistant amastigotes and promastigotes were examined by light microscopy and electron microscopy for the presence of any morphological differences from wild-type *L. mexicana*. No significant differences in size or shape of amastigotes or promastigotes were detected by light microscopy or in surface characteristics by scanning electron microscopy (not shown). However, a sig-

nificant feature was revealed by transmission electron microscopy: multilamellar membrane-like material was observed in the flagellar pockets of both AMB-resistant amastigotes and promastigotes (Fig. 1). This was a highly consistent feature present in 60 to 75% of thin sections through the flagellar pocket. Such material was never seen in wild-type *L. mexicana* and, interestingly, was also absent from AMB-resistant cells when grown in the absence of AMB. Thus, this material was specifically found in AMB-resistant cells exposed to AMB.

Infectivity and recovery of parasites from culture. In order to assess the likelihood of AMB-resistant parasites causing leishmaniasis in human patients, the ability of the AMB-resistant lines AR1 and PR1 to infect BALB/c mice was investigated, since this inbred strain is highly susceptible to leishmaniasis. AMB-resistant amastigotes were noninfective to mice, despite the injection of large numbers of parasites (3 × 10⁷ per mouse): lesions did not develop, and parasites could not be

TABLE 2. Endogenous sterol composition of various *L. mexicana* lines

Parasite	Sterol ^a	% Composition
Wild-type promastigotes	Ergosta-5,7,24(24 ¹)-trienol	85.1
	Ergosta-7,24(24 ¹)-dienol	4.2
	Stigmasta-5,7,24(24 ¹)-trienol	10.6
Resistant promastigotes	4,14,Dimethyl-cholesta-8,24-dienol	62.4
	4,14,Dimethyl-cholesta-7,24-dienol	6.5
	Lanosta-8,24-dienol	31.1
Resistant promastigotes grown for five passages without drug	4,14,Dimethyl-cholesta-8,24-dienol	91.0
	4,14,Dimethyl-cholesta-7,24-dienol	2.8
	Lanosta-8,24-dienol	6.2
Wild-type amastigotes	Ergosta-5,7,24(24 ¹)-trienol	40.9
	Ergosta-7,24(24 ¹)-dienol	4.6
	Stigmasta-5,7,24(24 ¹)-trienol	47.2
Resistant amastigotes	4,14,Dimethyl-cholesta-8,24-dienol	97.3
	4,14,Dimethyl-cholesta-7,24-dienol	1.4
	Lanosta-8,24-dienol	1.3
Resistant amastigotes grown for five passages without drug	4,14,Dimethyl-cholesta-8,24-dienol	80.9
	4,14,Dimethyl-cholesta-7,24-dienol	1.0
	14,Methyl-ergosta-8,24(24 ¹)-dienol	5.6
	Lanosta-8,24-dienol	8.6

^a All sterols are shown, except minor components comprising <0.1% of total.

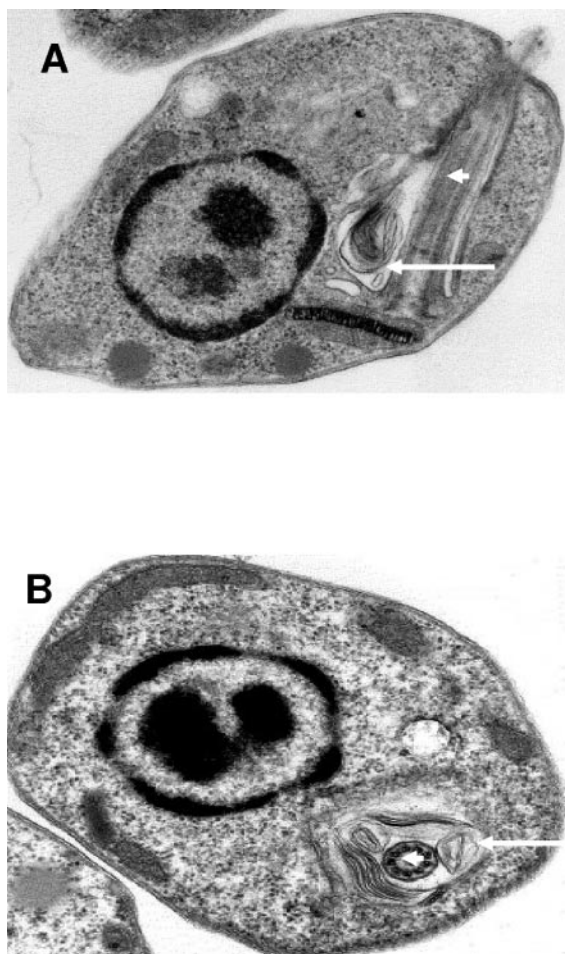


FIG. 1. Transmission electron micrographs showing (A) a longitudinal section through an AMB-resistant *L. mexicana* amastigote and (B) a transverse section through an AMB-resistant promastigote. Multilamellar membrane-like material in the flagellar pocket is indicated by arrows; the flagellum is indicated by arrowheads.

recovered by culture from the injection site or draining lymph nodes. However, injection of AMB-resistant promastigotes, even after 110 passages in vitro, did result in the development of cutaneous lesions, although these were much slower to develop than those from wild-type promastigotes. Amastigotes were isolated from these lesions and cultured axenically as amastigotes and promastigotes, and their AMB sensitivities were assessed. Both forms were highly resistant to AMB: amastigotes showed an IC_{50} of $2.2 \pm 0.62 \mu\text{g/ml}$, and promastigotes showed an IC_{50} of $4.25 \pm 0.33 \mu\text{g/ml}$. The latter value is particularly intriguing as this represents an apparent increase in resistance compared to that of the original infecting promastigotes (IC_{50} of $2.6 \mu\text{g/ml}$). Parasites reisolated from lesions initiated with drug-resistant promastigotes and grown as amastigotes both in the presence and in the absence of AMB showed sterol compositions similar to that of the original infecting AMB-resistant parasites (Table 2), indicating that the altered sterol composition was stable even after passage in vivo. The morphology of these parasites was also interesting. The AMB-resistant amastigotes were of normal appearance when isolated from lesions and behaved normally when cul-

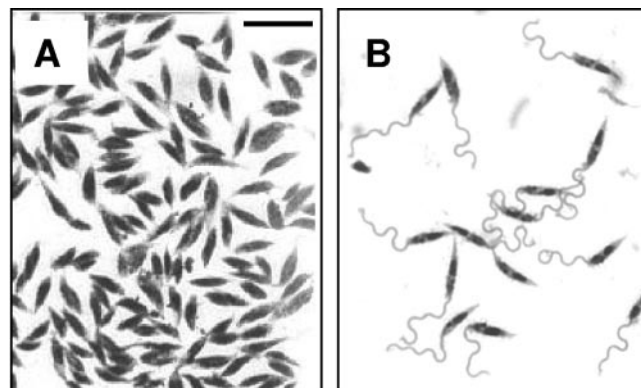


FIG. 2. Aflagellate AMB-resistant *L. mexicana* "promastigote" forms. (A) The culture shown was derived by an in vitro culture of AMB-resistant lesion amastigotes isolated from BALB/c mice under promastigote culture conditions in the presence of $4.0 \mu\text{g/ml}$ AMB. Bar, $10 \mu\text{m}$. (B) Control, wild-type promastigotes.

tured as axenic amastigotes, but when such amastigotes were incubated at 26°C to establish promastigote cultures, transformation was incomplete: nonmotile promastigote forms with very short or no flagella represented over 90% of the resulting stationary-phase population (Fig. 2). These aflagellate promastigotes grew readily and remained the dominant form in subsequent subpassages at 26°C . Reapplication of AMB selection to these new lines derived from AMB-resistant amastigotes produced amastigote and promastigote lines with even higher resistance to AMB, especially in aflagellate promastigotes, where growth in $16 \mu\text{g/ml}$ AMB was achieved.

Experimental infection and chemotherapy. The data described above show that stable AMB resistance can be generated in *L. mexicana* and that this property is retained following the infection of and recovery from BALB/c mice. However, it is also important to determine whether the drug resistance phenotype is exhibited in vivo, since this is where selective pressure would be exerted in the field. For this purpose, groups of BALB/c mice were infected with wild-type and AMB-resistant axenic amastigotes, and the responses of the mice to AMB chemotherapy were assessed. The AMB-resistant axenic amastigotes used in these studies were those derived from mouse infections established with promastigotes, as described in the previous section. Cutaneous lesions appeared later and increased in size more slowly in mice infected with AMB-resistant amastigotes than in mice infected with wild-type *L. mexicana* (Fig. 3). The wild-type parasites grew equally well in vitro, so the lower rate of lesion growth is presumably a product of the AMB-resistant phenotype that is exhibited only in vivo. When small lesions were apparent, chemotherapy was initiated; mice in each group were treated identically for a 2-week period as indicated. AMB treatment had a significant effect on wild-type-*L. mexicana* lesions: their rate of growth was lowered significantly ($P = 0.02$, Mann-Whitney test, week 10), and the lesions in the drug-treated subgroup remained significantly smaller until 14 weeks postinfection (Fig. 3A). In contrast, AMB treatment had no discernible effect on AMB-resistant-*L. mexicana* lesions (Fig. 3B), which remained similar to control, nontreated, AMB-resistant-parasite lesions for 22 weeks and beyond (not shown). Thus, although their lesion growth rate

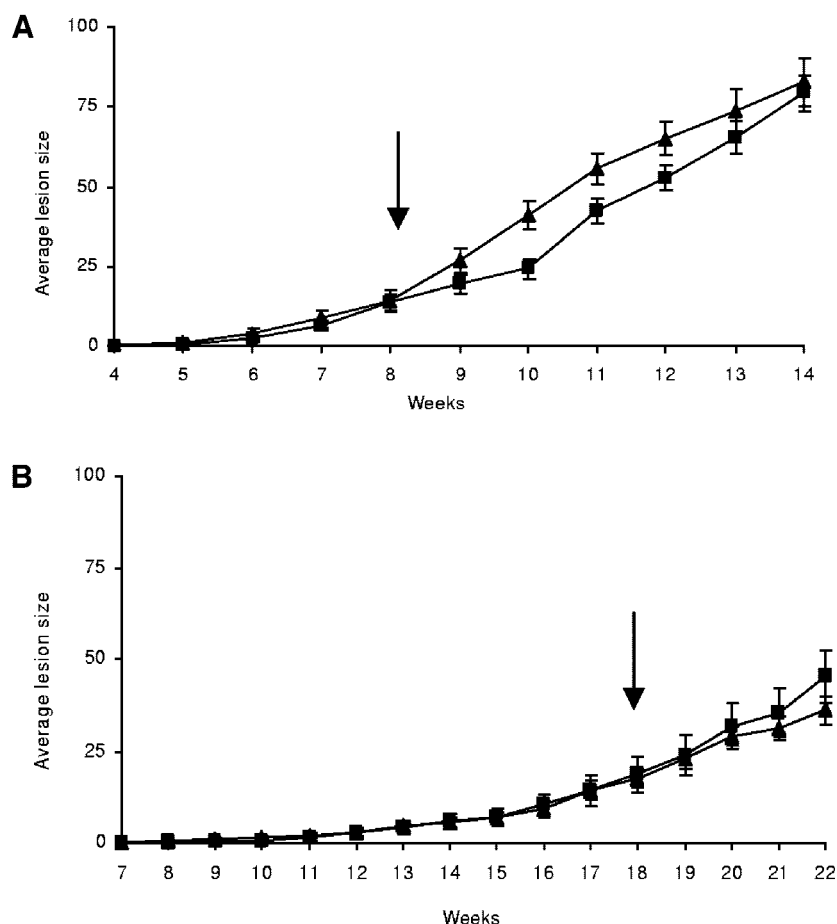


FIG. 3. Experimental chemotherapy of BALB/c mice infected with (A) wild-type and (B) AMB-resistant *L. mexicana*. Groups of eight to nine mice were either treated with 1 mg/kg AMB for a 2-week period (■), beginning at the points indicated by the arrows, or left as untreated controls (▲). Lesion size is length multiplied by width, expressed in mm.

was lower, AMB-resistant parasites were unaffected by AMB and maintained their phenotype in vivo.

DISCUSSION

In this report, we describe for the first time the response of *Leishmania* axenic amastigotes to AMB and the derivation of AMB-resistant amastigotes. The similarity in sensitivities of wild-type amastigotes and promastigotes of *L. mexicana* to AMB when similar conditions were used is an important result, as it indicates that, at least for this species, promastigotes are a suitable model for examining AMB resistance mechanisms. A key factor influencing AMB sensitivity in vitro was found to be the concentration of FCS in the culture medium. This effect probably has two components: AMB is not water soluble, so the protection afforded by FCS probably reflects sequestering of AMB by lipid components and/or albumin present in the serum, as well as the generally beneficial effects that higher concentrations of FCS have on parasite growth. It is also possible that higher levels of exogenous sterols from FCS could be protective.

The mode of action of AMB is believed to result from the interaction of AMB with membrane sterols, in which a com-

plex producing an aqueous channel spanning the membrane is formed (19, 31). This perturbation leads initially to increased membrane permeability to monovalent cations and then to other essential substances, resulting in disruption of cellular function. All eukaryotic cells contain sterols in their membranes, and the basis of the antifungal and antileishmanial selective toxicity of AMB is thought to be due to the preponderance of ergosta and stigmasterols (i.e., both are alkylated at C-24 in the side chain) in the surface membranes of these organisms, as opposed to the cholesta sterols typical of mammalian cells, and to the greater interaction between AMB and ergosta sterols. However, direct experimental proof for some of these assumptions is lacking, and a number of models have been proposed to explain the biological activity of AMB (5). Other mechanisms of action have also been suggested, including lipid peroxidation and stimulation (activation) of phagocytic cells such as monocytes (25). From the current study it is clear, however, that resistance of *L. mexicana* amastigotes to AMB is accompanied by marked changes in membrane sterol composition. These studies have shown an accumulation of methyl sterols, predominantly 4 α ,14 α -dimethylcholesta-8,24-dienol, in both highly resistant amastigotes and promastigotes. In contrast, Mbongo et al. (26) showed that, in

AMB-resistant *L. donovani* promastigotes, the normal membrane sterols were replaced by cholesta-5,7,24-trienol, a surprising result in view of the observation that AMB interacts most strongly with $\Delta^{5,7}$ sterols (14). These differing results may reflect differing levels of resistance in the various lines derived in the laboratory, since evidence has been obtained indicating that a sequence of different sterols predominated in AMB-resistant fungi as levels of resistance increased (15, 16). As resistance increased, there was a corresponding change in predominant sterol molecules, with a succession of $\Delta^{5,7}$, Δ^7 , and Δ^8 sterols being produced. This correlates with models of the interaction of AMB with sterols, since the double bonds in ring B of the sterol are expected to interact with the conjugated double bonds in the polyene. Only methylcholesta sterols were present in these resistant lines, so the lack of sterols alkylated at C-24 in the side chain may also be significant, though it is not clear whether a substitution at C-24 or the presence and positions of double bonds in the side chain are most important.

It is intriguing to see that the mutants described in the current study contain 14 α -methyl sterols. This type of sterol is also produced by the action of azole sterol biosynthesis inhibitors, such as ketoconazole (KTC), on C-14 demethylation and is expected, by analogy to studies of *Saccharomyces cerevisiae*, to have lethal effects by causing membrane disorganization. Equally interesting, the presence of C-4 methyl groups is also expected to be highly damaging, though again the parasites produced in our studies were able to survive and grow in their presence. The occurrence of C-4 gem-4 α ,4 β -dimethyl groups, or to some extent 4 α -methyl groups, may cause considerable steric interference with the sterol 3 β -hydroxyl group, which is thought to have a major role in the interaction of AMB with desmethyl sterols by hydrogen bonding to either the amino or the carboxyl group of AMB. In this way, C-4 methyl sterols could be protective against AMB action by replacing the sterols with which AMB will preferentially interact. It is interesting that, in the study of Ramos et al. (30), the sterol composition of *L. mexicana* was altered by the treatment with KTC, also to predominantly 4 α ,14 α -dimethylcholesta-8,24-dienol, and that these cells were resistant to the action of AMB, again indicating the lack of interaction of methyl sterols with AMB. The quantity of 4 α ,14 α -dimethylcholesta-8,24-dienol is remarkably consistent between the two studies: Ramos et al. reported values of 79.8 to 85.6% of endogenous sterols in KTC-exposed promastigotes (i.e., excluding exogenous cholesterol), compared to values of 62.4 to 97.3% from the current study (Table 2).

The generation of AMB-resistant *Leishmania* parasites with altered sterol compositions indicates that these parasites must have altered biosynthetic capabilities. Such alterations could include either upregulation or downregulation of specific enzymes or the pathway as a whole or could be due to specific mutations directly affecting the activity of enzymes. For example, it has been reported that AMB-resistant *L. donovani* has defective sterol 24-methyltransferase activity (29), that AMB-resistant *L. tarentolae* shows evidence of DNA amplification (34), and that defective enzymes exist in fungal pathogens (21).

In addition to their altered sterol compositions, the other significant feature of the AMB-resistant parasites was the presence of multilamellar lipid-like inclusions in the flagellar pocket. The role of these in resistance is not clear but was

strictly associated with the presence of AMB in the culture medium. This suggests a resistance mechanism in addition to altered sterol composition, such as an increased membrane turnover or perhaps some means of sequestering AMB from the surface and other cellular membranes. Interestingly, a similar feature was observed in some electron micrographs of *L. amazonensis* exposed to 22,26-azasterol, an inhibitor of sterol 24-methyltransferase (32).

The current study is the first in which AMB-resistant *Leishmania* parasites have been successfully used to infect animals. This is a significant finding, as it indicates that selection of viable AMB-resistant organisms could potentially occur in a mammalian host. The specific reasons why the promastigote lines retained infectivity whereas infectivity was lost by the amastigote lines are unknown, but this finding presumably reflects a more profound effect of long-term culture in the presence of AMB on amastigote infectivity than on that of promastigotes. Interestingly, the lesions produced by AMB-resistant parasites, whether resulting from the original promastigotes or the subsequent subpassage of resistant amastigotes, were always slower to develop than those of the wild-type controls. Presumably, the resistance phenotype was exacting some cost on the ability of amastigotes to replicate. The recovery from mice of parasites that retained their resistance phenotype was further proof of the stability of drug resistance. Significantly, the lines also exhibited their resistance phenotype under conditions of experimental chemotherapy. One intriguing feature of these mouse-derived lines in vitro was their reduced capacity for morphological transformation to promastigotes, which could be a factor impeding the acquisition of AMB-resistant parasites by sand flies and the subsequent development and transmission of resistant parasites by such infected flies.

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